NREL-Amoco CRADA Phase 3

Bench Scale Report 1.8

Scale-up of Pretreated Corn Fiber Batch SSCF with LNHST2

Project Title: Amoco-NREL CRADA with corn fiber

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Objective

Two batch SSCF (simultaneous saccharification and co-fermentation) runs with a mixture of pretreated corn fiber and corn screenings (20% total solids level) were performed to determine how reproducible the bench scale fermentation is and how is compares with large scale PDU fermentation runs.

Background

This experiment is designed to support the Task 3 PDU activities. Results obtained at the bench scale are to be compared to data obtained at the PDU scale to examine scaleability of lab data (from 1.7 L to 9,000 L). If the comparison is favorable, then bench scale can be used to collect critical data quickly and inexpensively.

Materials and Methods

Inoculum

The inoculum for each SSCF was generated in the PDU seed train. That train consisted of five stages starting with cultivation in two 250-mL Erlenmeyer flasks containing 50 mL of YEPD and progressing to an 160-L fermentor in the PDU. The inoculum for the first SSCF was generated with medium containing 5% w/v glucose. The first two stages in the laboratory were grown in YEPD (1% w/v yeast extract, 2% w/v peptone and 5% w/v glucose, pH 5.0) at 30°C and 150 rpm. The subsequent three stages performed in the PDU were grown in 1% w/v corn steep liquor (CSL) with 5% w/v glucose at pH 5.0. The inoculum for the second SSCF was generated in the same manner as the first SSCF except 2% w/v glucose was used in each stage instead of 5% w/v glucose due to slower inoculum growth associated the higher glucose concentration (see *PDU Run Report, CRADA Task #3*).

Pretreated** corn **fiber and corn screenings mixture

Pretreated biomass (a mixture of corn fiber and corn screenings) for the first **SSCF was** prepared in the APR.

The material for **the** bench-scale **SSCF** was collected **from** the APR while **PDU** vessel **450A was being** filled for the first time **during PDU Task** 3. The pH of this material was adjusted to 5.0 **with sodium** hydroxide, deionized water was added to bring the solids level **down,** and **an** amount of material needed to **yield** 20% solids (with the addition of enzymes, inoculum, **and** CSL taken into consideration) **in a 1.2-L final** volume fermentation was weighed **and** placed in a 1.7-L fermentor **and** autoclaved for one hour at 121°C.

The batch 2 SSCF was set-up at the bench scale **using** material retrieved **directly** from the PDU (450B, PDU run **4** in **Task** 3) fermentor after the enzyme, CSL and water had been **added** to minimize any differences between biomass processing at the bench scale and the **PDU.** Batch 2 was performed as a compliment to **PDU** 450B run 4.

Enzyme and nutrients

Cellulase and glucoamylase enzymes (see Report **1.7)** were added at 10 IFPU/g cellulose (based on a cellulose content of 17.3% in the **raw** feed **and** a cellulase activity of **70** IFPU/mL) and 2 IU/g cellulose **(based** on a starch content of 17.3% in the raw feed). The enzyme preparation used for the first batch SSCF was filter-sterilized through 0.2 µm filter before **being** added to the fermentor. Nisin (Alpin and Barrett Ltd, **England) was** added to both enzyme preparations at 200 mg/L as an antimicrobial agent (to prevent or suppress contamination) before **king** used in the second batch **SSCF.**

Corn steep liquor (1% w/v) was added as a nutrient source to each SSCF. To prepare the solution for the first SSCF, the pH of a 50% w/w solution of **CSL was** adjusted to 5.0 with **sodium** hydroxide pellets and autoclaved. As mentioned earlier, the material **used** in batch **2** already contained CSL.

*Sampling **and** Analysis*

Initial **and** final samples were obtained for **a** total compositional analysis. Samples were also taken **during** the course of the experiment, **and** the liquid fraction was analyzed for cellobiose, succinic acid. lactic acid, glycerol, acetic acid. HMF, furfural, **and** the monomeric **and** oligomeric sugars glucose, xylose, galactose, arabinose, **and** mannose. Colony forming **units** were monitored on each sample to **measure** cell growth.

Results and Discussions

First Batch SSCF

The major available sugars for use by LNHST2 in this **fermentation are** monomeric and oligomeric (including cellobiose) glucose in the liquor and cellulose in the solids, as **well as** the monomeric xylose found in the liquor. Table 1 summarizes the **initial** and final glucose **and** xylose levels in both the liquor **and** the solid fractions in batch 1 **and** batch **2** SSCF. Within 18.5 hours of SSCF, a majority of the **initial** monomeric glucose was consumed (Figure 1). Of the total available glucose, 74.09% was consumed in **113** hours. Of the glucan in **the** solids, **75.4% was** either consumed **by** LNHST2 or converted into oligomeric glucose. Figure 2 shows that the oligomeric concentration increases throughout the fermentation **as** the glucan level decreases. This demonstrates that

cellulase is actively breaking down the cellulose fraction, but that cellobiose is accumulating. The rate of glucose utilization was 3.523 g/L-h.

After an initial phase of slow uptake of xylose (–12 hours while a majority of glucose was being consumed), the rate of xylose utilization increased to 0.912 g/L-h. Interestingly. after the monomeric glucose was consumed, the xylose rate decreased to 0.270 g/L-h. By 84 hours, a majority of the monomeric xylose was consumed By the end of the SSCF. 92.8% of the monomeric xylose had been consumed (62.81% of the total available xylan).

Table 1: Initial and Final Glucose and Xylose Concentrations and Conversion

		Batch 1			Batch 2		
		t_0 (g/L)	t_f* (g/L)	convesion (%)	b (g/L)	t_f* (g/L)	conversion (%)
Equivelant Glucose							
soluble	monomeric	50.13	1.69		51.41	1.98	
	oligomeric	10.55	12.39		6.81	9.87	
	cellobiose	2.82	2.79		5.84	1.97	
insoluble	glucan	31.89	7.85	75.38	29.50	6.40	7S.30
	Total	95.39	24.72	74.09	93.56	20.22	78.39
Equivelant Xylose							
soluble	monomeric	27.96	2.01	92.81	26.98	8.62	68.05
	oligomeric	12.08	12.55		8.08	7.01	
insoluble	xylan	1.21	0.78		1.75	0.53	69.9
	Total	41.25	15.34	62.81	36.81	16.16	56.10
Glucose and Xylose							
	Total	136.64	40.06	70.68	130.37	36.38	72.09

* The duration (t_f) of each run was 113 h for batch 1 and 167 h for batch 2.

During the SSCF, 39.74 g/L of ethanol were produced corresponding to an ethanol metabolic yield of 80.5% of theoretical (based on the consumed xylose and glucose) (Table 2). The ethanol process yield was 56.910 of theoretical (based on the total sugars available). About 25.4 g/L of total glucose and monomeric xylose remained unused by the end of the fermentation representing a potential for 12.95 g/L more ethanol through process and/or microorganism optimization.

Table 2: Performance Parameters of SSCF by LNHST2

Parameter	Value	
	Batch 1	**Batch 2**
Glucose conversion	74.09%	78.39%
Xylose Conversion	62.81%	**56.10%**
Ethanol Process Yield (% theoretical)	***56.9%***	63.50%
Ethanol Metabolic Yield (% theoretical)	80.5%	88.07%

Table 3 lists the products and their yields based on the consumed sugars in the fermentation. The major by-product was glycerol at 0.045 g/g consumed sugars and xylitol at 0.026 g/g consumed sugars. The cell mass yield was based on an estimated yield of –0.05 g/g consumed glucose; since cell mass quantification in the presence of insoluble solids is difficult and unreliable. Previous studies on pure sugars indicated that the cell mass yield can vary from 0.04 to 0.10 g/g of consumed sugars. The overall mass balance closure was 92.37%.

Table 3: By-product and Carbon Balance for Batch 1 and 2 SSCF

	g produced 100 g consumed glucose and xylose	
Products	**Batch 1**	**Batch 2**
Ethanol	**41.14**	**45.00**
Cell Mass	4.04	3.94
Carbon Dioside	39.35	43.05
Glycerol	**4.47**	5.02
Aceuc Acid	0.32	0.00
Lactic Acid	0.00	0.17
Succinic Acid	0.45	0.55
Xylitol	2.61	2.75
Total	92.37	100.48

***Second** Batch SSCF*

A second batch SSCF was performed with material processed in the PDU (pH adjusted, CSL and enzymes added, and water added to bring the total solids to the appropriate operating level) to minimize any differences between the material used for bench scale and PDU experiments. The pretreatment process liberated 64.06 g/L of soluble glucose (monomeric and oligomeric) and 26.98 g/L of monomeric xylose, which are similar to the levels in batch 1 (63.50 g/L and 27.96 g/L, respectively). After a lag of 6 h, glucose was used at a rate of 2.41 g/L-h. Within 24 h of inoculation, all of the monomeric glucose was consumed (Figure 3). After that, the rate-limiting step was the liberation of glucose from oligomers and cellulose by the catalytic action of cellulase. The overall glucose conversion, including the total soluble and insoluble glucan fractions, was 78.39% after 167 hours in batch mode (Table 1).

of the 26.98 g/L monomeric xylose available, 18.36 g/L was consumed, representing a conversion of 68.1% (Table 1) (56.7% conversion of the total xylan). The conversion rate for xylose was 0.249 g/L-h. The phenomena observed in the first batch, where xylose consumption was higher in the presence. of glucose, was not apparent in batch 2. It should be noted that in the batch 1 SSCF, 92.8% of the monomeric xylose was consumed. The major difference between the two studies was the amount of acetic acid produced during pretreatment (2.52 g/L in batch 1 compared to 4.93 g/L in batch 2) (Figure 4). The inhibitory effect of high acetic acid concentration on xylose utilization and ethanol production has been confirmed by studies performed with a variety of yeast strains at various pH levels (Van Zyl et al 1991)*. Hence, the incomplete use of monomeric xylose by LNHST2, as evidence by the process yield of 63.50%, could be attributed to the detrimental effect of acetic acid inhibition (and perhaps other inhibitors) on cell metabolism. Nevertheless, even though not all the xylose was consumed, the ethanol metabolic yield was high, 88.07% (Table 2) or 0.450 g ethanol per gram of consumed sugars.

In addition to the decrease in xylose utilization, the cell concentration was also lower in Batch 2 compared to Batch 1 (Figure 5). The cell mass in batch 1 increased to 1.07×10^8 cells/mL by 18.5 h. where it remained during the fermentation. In batch 2. the cell population reached a maximum of 6.8×10^7 cells/mL at 25 h into the fermentation and then declined. Again. inhibition of cell metabolism may be the reason.

The distribution of by-products was very similar to that observed in batch 1. As shown in Table 3, glycerol was the major by-product at 0.050 g/g consumed sugars. The overall mass balance closure was very satisfactory at 100.48%.

Correlation between PDU and bench scale run

The pretreated biomass was also subjected to SSCF in a 9.000-L pilot plant fermentor under conditions similar to those at the bench scale. As seen in Figure 3. the results of the pilot plant run correlated very well with the bench-scale data. After 24 hours of SSCF. the concentrations of glucose. xylose. and ethanol were 2.7 g/L. 24.9 g/L. and 29.4 g/L in the bench scale fermentor and 3.7 g/L, 23.8 g/L. and 31.1 g/L in the pilot fermentor. At 96 hours, the concentrations of these same components were 1.5 g/L. 11.9 g/L, and 40.5 g/L in the bench reactor and 1.7 g/L. 9.4 g/L. and 43.4 g/L in the pilot-scale reactor. Similarly, by-product formation was in close agreement. as indicated by xylitol, which reached 2.4-2.5 g/L in both vessels (data not shown). The good correlation shows that despite the 5,300-fold increase in fermentor size. the performance of ST2 remained unchanged and very promising. The scaleability and reproducibility of the SSCF performance justify further optimization studies for use of the recombinant cofermenting yeast in commercial applications.

Conclusions

The data obtained from the two bench scale fermentations correlate quite well, as the total glucose and xylose consumption was 71.87% and 72.98% in batch 1 and batch 2, respectively. The slightly lower xylose consumption in batch 2 seems to be the result of cell inhibition by acetic acid and ethanol. The presence of other inhibitors and possible nutrient limitations may have also played a role in that phenomenon. Finally, the data from the bench scale batch 2 and the PDU 450B run 4 correlate very well indicating that the bench scale results are readily scaleable.

*Van Zyl, C., Prior, B. A., Du Preez, J. *Enzyme Microb. Technol.* 13, 82-86. (1991).

Figure 1: SSCF Batch 1 with LNHST2
(Task 3 Bench Scale Data Run 1, PDU Run 1)

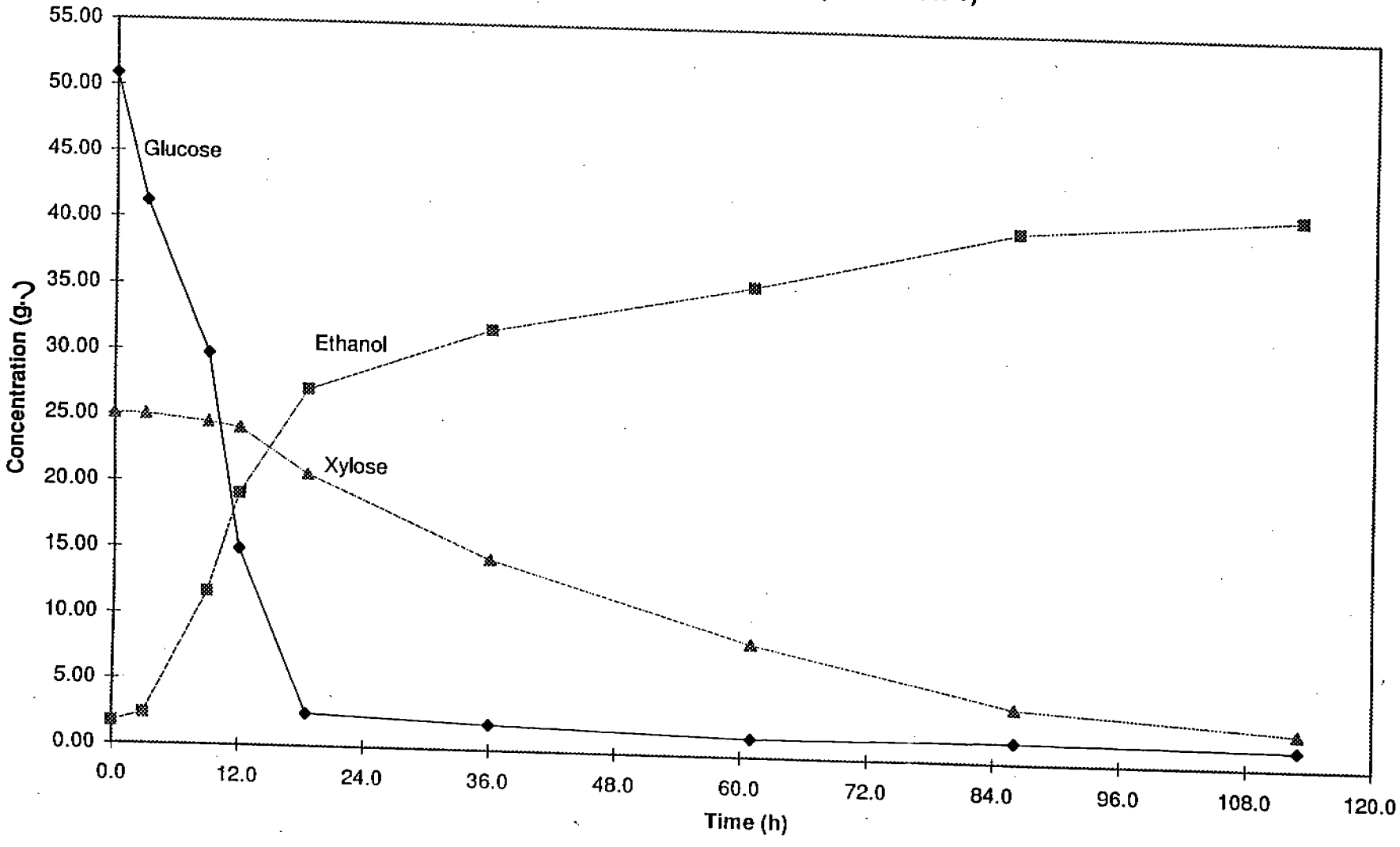
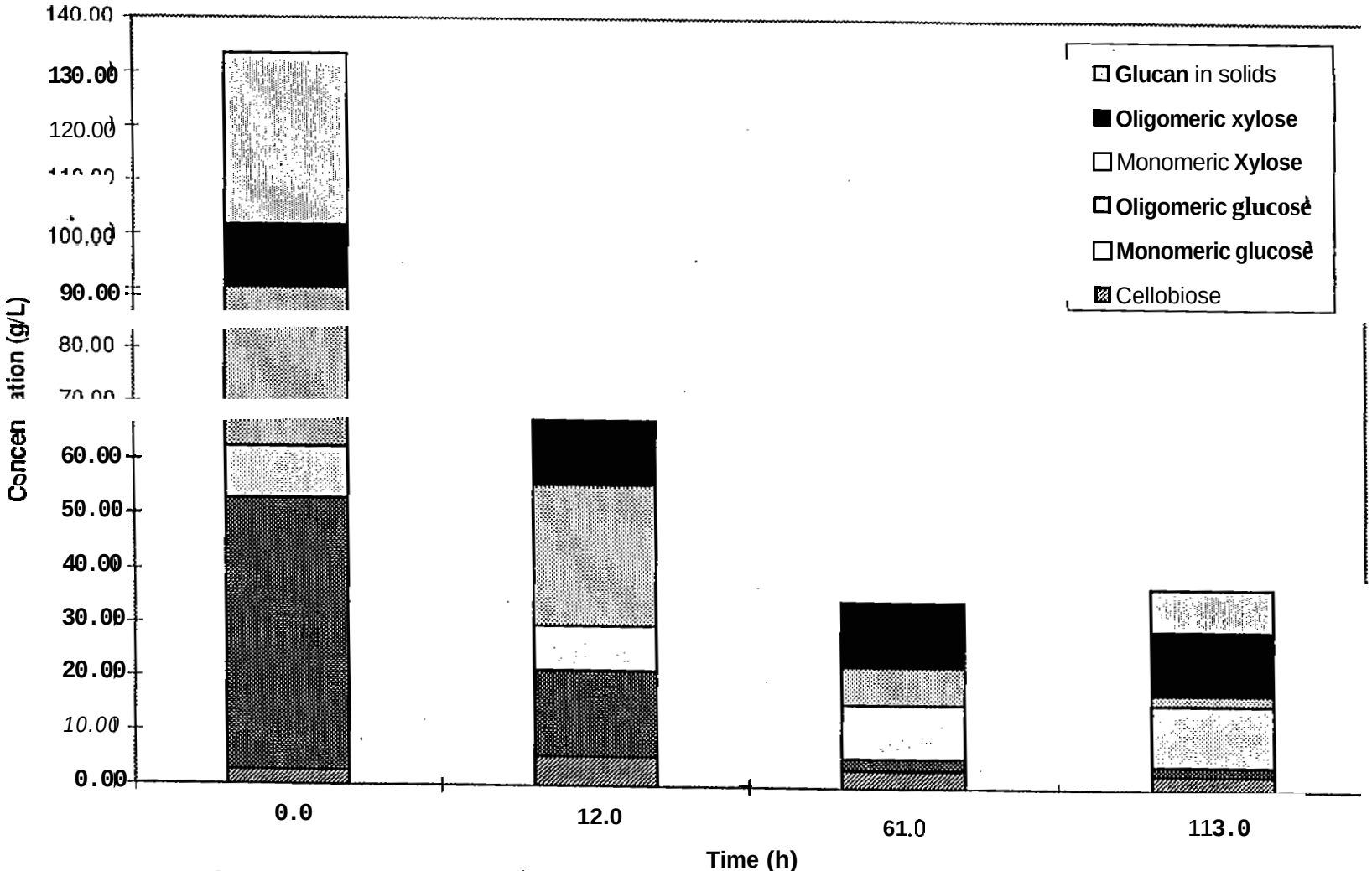


Figure 2: Batch 1 SSCF Sugar Consumption



Solids glucan only **reported at 0 and 113 hours**

Figure 3: Comparison of Bench Scale and PDU Scale SSCF Results with LNHST2

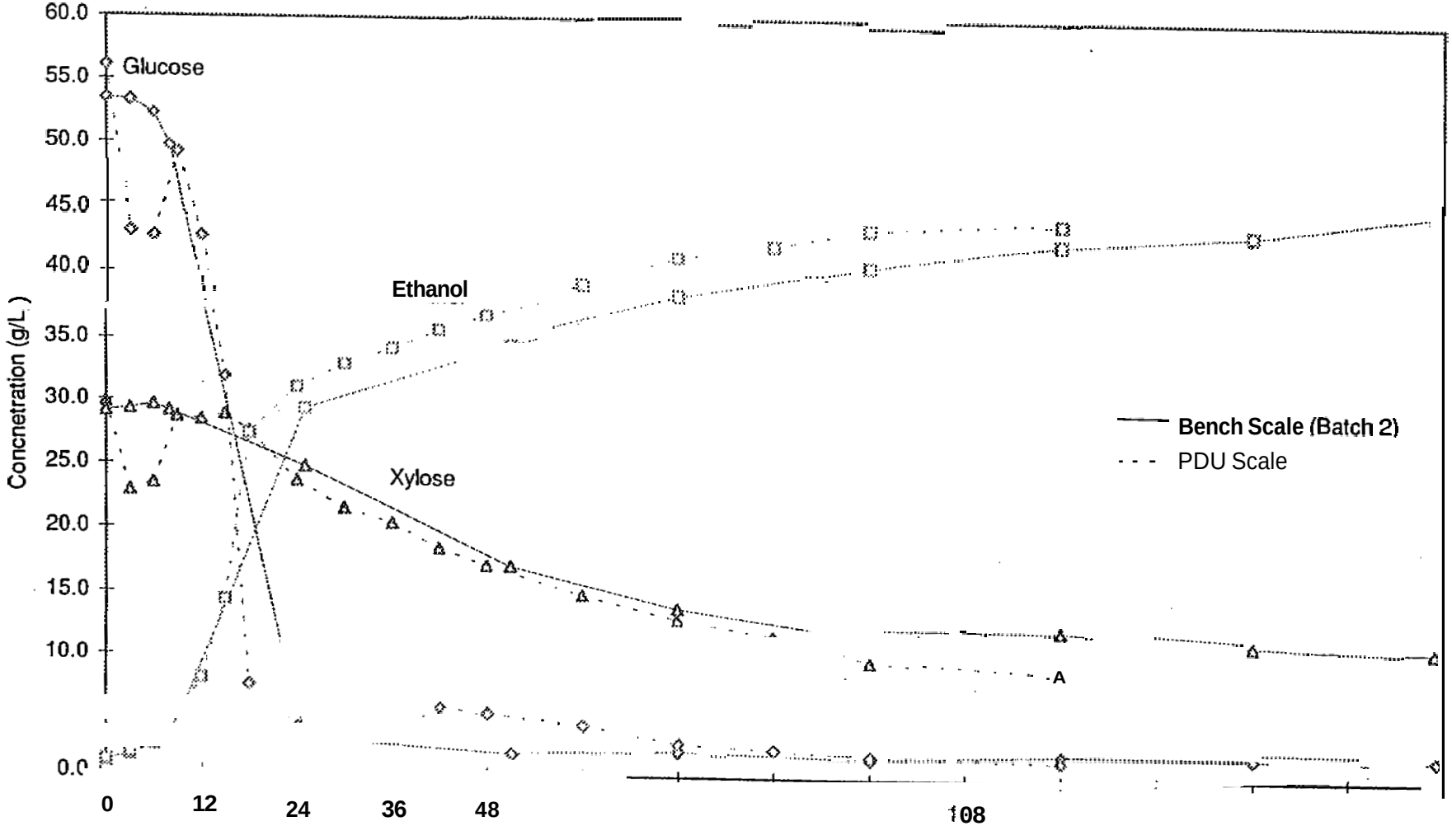


Figure 4: By-products from SSCFs with LNHST2
(Comparison of Bench Scale Data Runs 1 and 2)

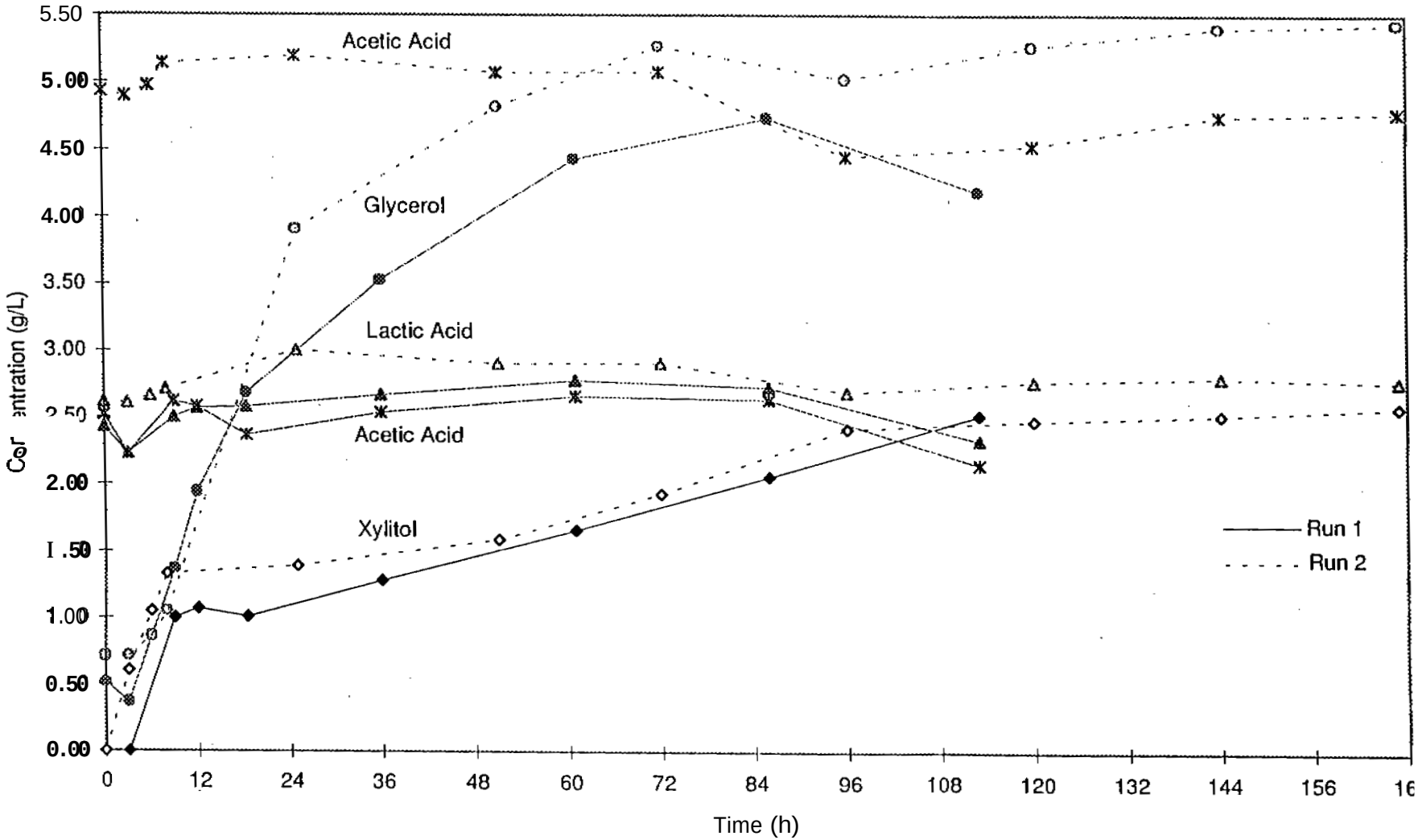
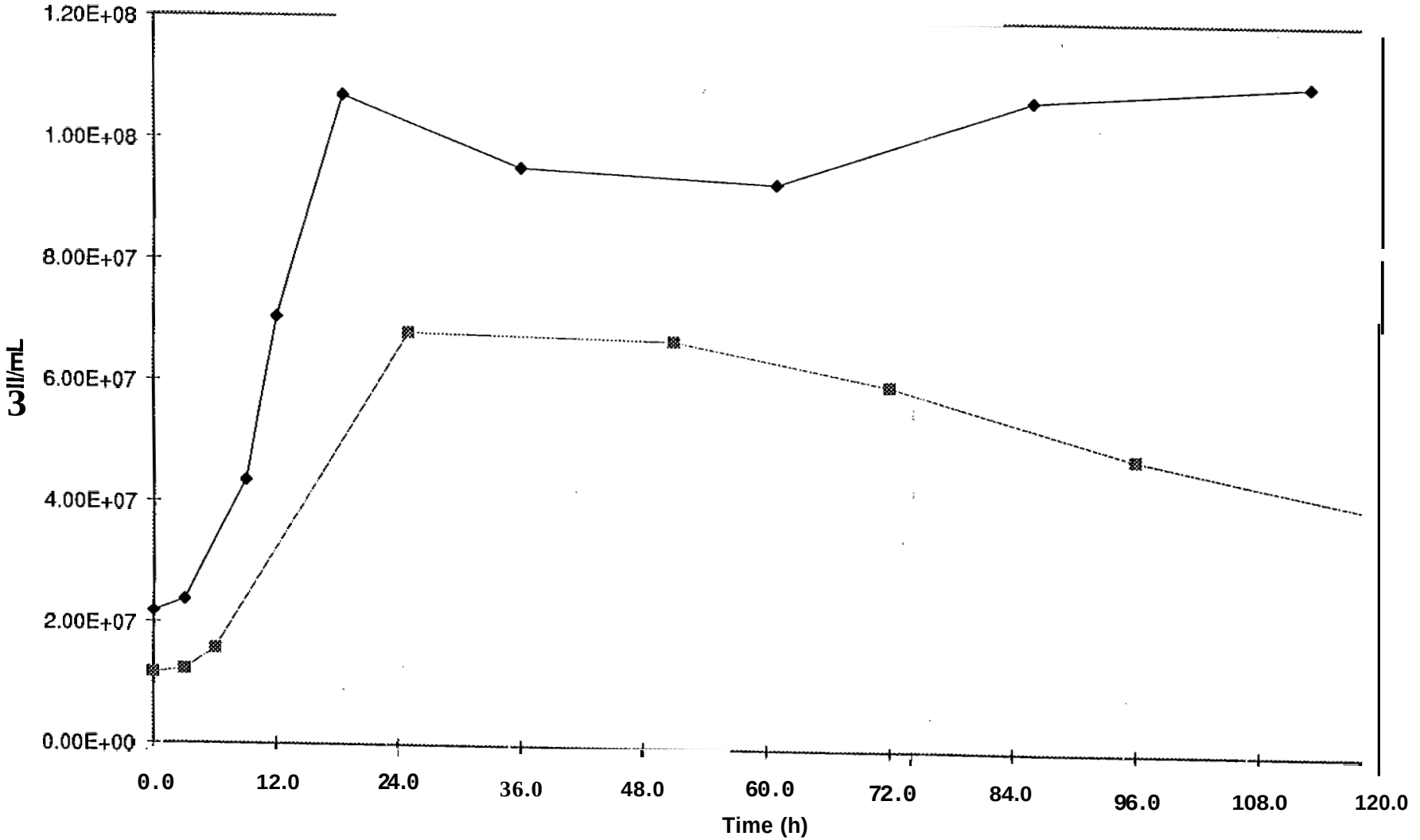


Figure 5: Comparison of Colony Forming Units Between Batch One and Two SSCF



Raw Data

(Batch 2 in **Report** 1.0) CAT Task Analysis No. 96-028 (liquors) and 96-029 (solids)

	Tracking samples performed by NREL						By-products						
	YSI	LC	LC	YSI	LC	GC	LC						
Sample	Time (h)	Glucose (g/L)	Glucose (g/L)	**Xylose** (g/L)	Ethanol (g/L)	Ethanol (g/L)	Ethanol (g/L)	Cellobiose (g/L)	Xylitol (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	**Glycerol** (g/L)	**Acetic** acid (g/L)	Ethanol (g/L)	CFU/mL
1	0	48.88	53.45	29.12	1.36	0.92	1.10	5.36	0.00	0.00	2.61	0.71	4.93	1.10	1.17E+07
2	**3**	50.00	53.31	29.33	1.88	1.20	1.40	5.74	***0.61***	0.19	2.61	0.72	4.89	1.40	1.22E+07
3	6	47.88	52.25	**29.63**	2.58	**2.09**	**2.10**	6.08	1.05	0.28	2.66	0.87	4.97	2.10	**1.57E+07**
4	8	46.63	49.70	**29.16**	3.13	2.89	2.90	6.21	1.33	0.56	2.71	1.05	**5.13**	2.90	
5	25	0.60	2.70	24.86	28.63	29.39	27.40	3.99	1.39	0.61	3.00	3.91	5.19	27.40	**6.80E+07**
6	51	0.46	1.71	17.21	33.13	34.66	36.60	3.02	1.59	0.55	**2.90**	4.82	5.07	**36.60**	6.70E+07
7	72	0.45	1.96	14.03	37.21	30.07	36.00	2.65	1.93	0.71	2.91	5.26	5.07	**36.00**	**6.00E+07**
8	96	0.45	1.53	11.85	38.63	40.48	30.60	2.30	2.42	0.47	2.69	5.02	4.45	38.60	**4.83E+07**
9	120	0.41	2.00	11.73	39.50	42.40	40.20	2.47	2.48	0.51	2.77	5.26	**4.53**	40.20	**4.00E+07**
10	144	0.41	1.99	10.79	39.90	43.25	41.50	2.44	2.52	0.49	2.80	5.40	4.75	41.50	1.70E+07
11	167	0.39	**1.88**	10.45	39.82	**45.09**	43.40	2.34	2.58	0.52	2.77	5.43	**4.78**	43.40	1.40E+07

(Batch 1 In Report 1.8) **CAT Task** Analysis No. 96-025 (liquors) and 96-027 (solids)

	Tracking samples performed by NREL						By-products						
	YSI	LC	LC	YSI	LC	GC	LC						
Sample	Time (h)	**Glucose** (g/L)	**Glucose** (g/L)	**Xylose** (g/L)	Ethanol (g/L)	Ethanol (g/L)	Ethanol (g/L)	Cetlobiose (g/L)	Xylitol (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (g/L)	CFU/mL
1	0.0	42.50	50.87	25.10	1.90	1.69	0.90	2.68	0.00	0.00	2.43	0.52	2.52	0.90	2.18E+07
2	**3.0**	43.90	41.21	**25.05**	2.77	2.34	2.60	3.00	0.00	0.00	2.23	0.37	2.23	2.60	**2.37E+07**
3	**9.0**	31.00	29.75	24.53	13.10	11.68	11.40	4.05	1.00	0.31	2.50	1.37	2.62	11.40	**4.35E+07**
4	12.0	11.40	14.97	24.09	20.80	19.12	18.30	**4.26**	1.07	0.36	2.57	1.94	2.58	18.30	**7.05E+07**
5	18.5	0.40	2.46	20.67	27.80	27.10	25.60	3.11	1.01	0.34	2.58	**2.68**	2.37	25.60	**1.07E+08**
6	36.0	0.37	1.94	14.50	**31.58**	31.86	29.80	2.46	1.28	0.42	2.67	**3.53**	2.54	29.80	**9.53E+07**
7	61.0	**0.23**	1.41	**8.55**	38.34	35.54	**33.30**	2.63	1.66	**0.46**	2.78	4.43	2.66	33.30	**9.30E+07**
8	**86.0**	0.20	**1.61**	4.13	**39.98**	40.01	36.90	1.50	2.06	0.45	2.73	4.74	2.64	**36.90**	1.07E+08
9	113.0	0.18	t.39	2.67	39.22	41.42	37.90	1.52	2.52	0.42	2.33	**4.19**	2.15	**37.90,**	1.10E+08

Raw Data

Batch 2

Monomeric and Oligomeric Sugars Analysis

Cellobiose (g/L)	Monomeric Glucose (g/L)	Oligomeric Glucose (g/L)	Total soluble glucose (g/L)	Monomeric Xylose (g/L)	Oligorneric Xylose (g/L)	Total soluble xylose (g/L)	Monomeric galactose (g/L)	Oligomeric galactose (g/L)	Total soluble galactose. (g/L)	Monomeric arabinose (g/L)	Oligomeric arabinose (g/L)	Total soluble arabinose (g/L)
5.55	51.41	6.13	63.09	**26.98**	7.57	34.55	6.54	0.59	7.13	20.75	1.08	**21.83**
5.96	50.44			26.95			6.79			**20.60**		
6.21	48.97			26.46			6.48			**20.54**		
6.39	**47.46**			26.78			6.53			20.74		
3.79	4.52	10.48	**18.79**	22.71	7.98	**30.69**	6.56	**0.98**	**7.54**	18.72	2.32	**21.04**
2.53	3.10	**11.00**	16.63	16.00	**7.81**	23.89	6.82	0.66	7.48	**18.18**	**2.31**	**20.49**
2.43	2.73	**11.58**	16.74	12.67	7.31	19.98	7.09	**-0.03**	7.06	18.17	**I.76**	**19.93**
2.08	2.51	10.44	15.03	**10.50**	7.62	18.12	**4.67**	1.12	**5.79**	17.46	3.61	**21.07**
2.03	2.29	**9.92**	14.24	9.65	7.21	16.86	4.75	1.00	5.75	17.42	**3.43**	20.85
1.94	0.44	11.25	13.63	**8.97**	7.12	16.09	**4.82**	**0.85**	5.67	17.20	**4.36**	21.56
1.87	1.98	**8.88**	**12.73**	8.62	**6.56**	15.18	**4.85**	0.68	5.53	**17.07**	3.65	**20.72**

Batch 1

Monomeric and Oligomeric Sugars Analysis

Cellobiose (g/L)	Monomeric Glucose (g/L)	Oligorneric Glucose (g/L)	Total soluble glucose (g/L)	Monomeric Xylose (g/L)	Oligomeric Xylose (g/L)	Total soluble xylose (g/L)	Monomeric galaclose (g/L)	Oligorneric galactose (g/L)	Total soluble galactose (g/L)	Monomeric arabinose (g/L)	Oligorneric arabinose (g/L)	Total soluble arabinose (g/L)
2.68	**50.13**	**9.50**	**62.31**	27.96	11.31	39.27	5.93			18.21	5.04	23.25
5.93	**40.2**			**27.53**			5.95			**17.87**		
5.98	29.72			**26.86**			5.83			17.50		
5.4t	15.78	**8.36**	**29.55**	26.01	11.86	37.87	5.75			16.99	3.43	20.42
3.58	4.92			18.61			5.45			15.27		
3.16	**3.73**	**7.48**	14.37	12.72	**6.1**1	18.83	5.15	0.79	**5.94**	15.08	1.38	**16.46**
3.19	2.2	**9.83**	15.22	7.01	12.15	**19.16**	**5.10**	0.52	**5.62**	**14.33**	**4.15**	**18.48**
3.06	2.03			3.04			**5.09**			14.32		
2.65	1.69	11.15	15.49	2.01	11.75	13.76	4.95	0.17	5.12	13.96	**3.86**	17.82

Raw Data

Batch 2

[redacted]

[redacted] Solids Ana ysis (% Dr /

Monomeric mannose (g/L)	Total soluble mannose (g/L)	Glucose	Xylose	Galactose	Arabinose	Mannose	Klason Lignin	Acid Soluble Lignin	Total Ash	Total Solids	HMF (g/L)	Furfural (g/L)
0.00	0.05	40.18	2.38	0.40	2.04	0.00	32.99	7.46	1.67	22.20	0.27	0.33
0.00											0.26	0.28
0.00											0.25	0.19
0.00											0.22	0.14
0.00	0.11										0.00	0.14
0.00	0.08										0.00	0.73
0.00	0.09										0.00	0.26
0.00	0.07										0.00	0.00
0.00	0.06										0.00	0.00
0.00	0.09										0.00	0.00
0.00	0.10	13.40	1.10	0.20	0.80	0.00	46.81	10.29	0.52	22.46	0.00	0.00

Batch 1

[redacted] **Solids Analysis (% Dry weight)** [redacted]

Monomeric mannose (g/L)	Total soluble mannose (g/L)	Glucose	Xylose	Galactose	Arabinose	Mannose	Klason Lignin	Acid Soluble Lignin	Total Ash	Total Solids	HMF (g/L)	Furfural (g/L)
0.00	0.00	55.2	2.1	0.1	2.0	0.0	20.1	6.69	0.3	11.2	0.22	0.18
0.00											0.21	0.13
0.00											0.00	0.00
0.00	0.00										0.09	0.00
0.00											0.00	0.00
0.00	0.00										0.08	0.00
0.00	0.00										0.00	0.00
0.00											0.00	0.00
0.00	0.00	20.62	2.04	0.15	1.04	0.00	3636.00	8.39	0.90	22.01	0.00	0.00